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Chunghyuldan Downregulates the Activation of Transcription Factors NF-κB and AP-1 of BV-2 Cells Induced by Lipopolysaccharide

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Abstract – Chunghyuldan (Qingxuedan *in Chinese*) (CHD) has been used for patients with atherosclerosis and brain ischemia in Korea. To evaluate antiischemic activity of CHD, its antiinflammatory effect in lipopolysaccharide-induced BV-2 cells was investigated. CHD potently inhibited nitric oxide (NO) production in LPS-induced BV-2 cells with an IC_{50} value of 4.8 μg/ml. CHD did not only inhibit mRNA and protein expression levels of inducible NO synthase and cyclooxygenase-2 in LPS-induced BV-2 cells, but also repressed mRNA expression levels of proinflammatory cytokines IL-1β and TNF-α. CHD also downregulated the activation of NF-κB and AP-1 transcription factors induced by LPS. These results suggest that CHD may improve inflammatory brain ischemia by the downregulation the activation of NF-κB and AP-1 transcription factors.

Key words □ Chunghyuldan, Qingxuedan, NF-κB, AP-1, BV-2 cell

INTRODUCTION

An inflammatory reaction in the brain, due to intervention or decrease of blood circulation, causes an ischemic stroke, and occurs in about 80% of brain stroke patients (Barber *et al.*, 2003; Bartosik-Psujek *et al.*, 2003). The inflammatory responses are mainly modulated by activated neutrophils and macrophages (Saleh *et al.*, 2004; Khan *et al.*, 2004). Nitric oxide (NO) and prostaglandins are two pleiotropic mediators produced at inflammatory sites by the enzymes constitutive and inducible NO synthases, cyclooxygenase (COX)-1 and COX-2 (Appleton *et al.*, 1996). Stimuli such as cytokines and/or bacterial lipopolysaccharide (LPS) induce inducible NO synthase (iNOS) protein expression (Dubois *et al.*, 1998; MacMicking *et al.*, 1997; Perkins and Kniss, 1999; Smith *et al.*, 1996; Stefanovic-Racic *et al.*, 1993). The gene expressions of these enzymes are regulated by transcription factors, such as NF-κB and AP-1 transcription factors, on macrophagaes stimulated with LPS (Dubois *et al.*, 1998; Valles *et al.*, 2004).

Chunghyuldan (Qingxuedan *in Chinese,* CHD), which consists of Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, Gardeniae Fructus and Rhei Rhizoma, is known to have antihyperlipidemic activities,[12,13] and has been used for patients with atherosclerosis and brain ischemia (Kim *et al.*, 2002; Jung *et al.*, 2003; Cho *et al.*, 2005). Recently, we reported that CHD inhibit COX-2 and iNOS protein expression in LPS-induced RAW264.7 cells (Cho *et al.*, 2004). Nevertheless, CHD did not clarify the mechanism of the enzyme gene regulation.

Therefore, in present study, the effect of CHD on the activation of NF-κB and AP-1 transcription factors in LPS-induced BV-2 microglial cells was investigated.

MATERIALS AND METHODS

Materials

The Griess reagent was purchased from Promega Co. (Madison, WI, USA). The anti-rabbit inducible NO synthase (iNOS),

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anti-goat COX-2, anti-rabbit nuclear factor-κB (NF-κB), and anti-mouse β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, U.S.A.). RNeasy Protect Mini kit was from Qiagen Co. (Valencia, CA, USA). AccPower® RT/PCR Premix was from Bioneer Co. (Seoul, Korea).

CHD was prepared according to the previous method (Kim *et al.*, 2002). It consists of 80% EtOH extracts of Coptidis Rhizoma 4 g, Phellodendri Cortex 4 g, Scutellariae Radix 4 g, Gardeniae Fructus 4 g, and Rhei Rhizoma 4g.

BV-2 microglial cell culture

The immortalized murine BV-2 cell line that exhibits both the phenotypic and functional properties of reactive neuronal microglia cells were cultured in the Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotics (Bocchini *et al.*, 1992). The cultures were maintained at 37°C in humidified 5% CO_2 and split once per two days. In all experiments, CHD was treated 30 min before the addition of LPS in DMEM. CHD was dissolved in DMSO and the final concentration of DMSO added to cells never exceeded 0.1%.

Immunoblot analysis of iNOS, COX-2, NF-κB and I-κB in LPS-induced BV-2 cells

Immunoblot analyses of the iNOS, COX-2 and NF-kB were performed according to the method of Ishihara *et al.* (2000). BV-2 cells (1.5×10^6 cells on 60 mm dish) were incubated for a day. Then, cells were pretreated with test materials. After 60 min pretreatment, cells were stimulated with LPS (1 μg/ml) for 6 h (in NF-κB and I-κB) or 16 h (in iNOS and COX-2). The cells were washed in 2-3 ml of ice-cold PBS, centrifuged at 1000 × g for 5min. Then, cells were lysed in lysis buffer [10 mM Tris (pH 8.0), 140 mM NaCl, 1% Triton 0.5% SDS, 10% protease inhibitor (Complete Mini, Germany)] and centrifuged at 12000 × g and 4°C for 15 min. The supernatant was used as the cytosol fraction for the immunoblot assays for the iNOS, COX-2 and I-κB protein expressions. The pelleted nuclei fractions for the immunoblot assays of the NF-kB protein expression were resuspended in the extraction buffer, containing 10 mM Tris (pH 8.0), 50 mM KCl, 300 mM NaCl, 1 mM dithiothreitol, 5 μg/ml pepstatin A and 5 μg/ml aprotinin, and then lysed on ice for 30 min. The lysed nuclei fraction was centrifuged at 12000 × g and 4°C for 30 min. The resulting supernatant was used as a nuclei fraction for the analysis of NF-κB protein expression level. Protein expression levels of COX-2, iNOS, NF-κB, I-κB and β-actin of the cell lysates (50 μg) were analyzed by the above immunoblot method.

RT-PCR analysis

BV-2 cells (1.5×10^6 cells on 60 mm dish) were treated with LPS (1 μg/ml) in the presence or absence of CHD for 6 h and total RNA was isolated using the RNeasy Protect Mini kit. The RT-PCR was performed with AccPower® RT/PCR Premix (Bioneer, Seoul, Korea) (Chi *et al.*, 2003). The primers were designed as described by UniSTS database: COX-1, forward primer 5'-CTTTTATCCTCCCAGGATTTGG-3' and reverse primer 5'-GCTAAATACTTTGACACCGG-3' (product size 231 bp); COX-2, forward primer 5'-TGTATCCCCCCACAGTCA AAGAC-3' and reverse primer 5'-GTGCTCCCGAAGCCA-GATGG-3' (product size 146 bp); iNOS, forward primer 5'-GTGAGGATCAAAAACTGGGG-3' and reverse primer 5'-ACAACGTGGAGAAAACCCCAGGTG-3' (product size 380); IL-1β forward primer 5'- ATGGCAACTGTCCCTGAACT-3' and reverse primer 5'- GTCGTTGCTTGTCTCTCCTT-3' (product size 508 bp); tumor necrosis factor (TNF)-α, forward primer 5'- CCCTCACACTCAGATCATCTTCTCAA-3' and reverse primer 5'- TCTAAGTACTTGGGC AGGTTGACCTC-3' (product size 425 bp); GAPDH, forward primer 5'-ACCA-CAGTC CATGCCATCAC-3' and reverse primer 5'-TCCAC-CACCCTGTTGCTGTA-3' (product size 452 bp). The amplification was performed at 94°C for 30 - 60 s, and 55 - 62°C for 15 - 40 s, and 72°C for 30 - 60 s with 32 cycles for COX-2 and 30 cycles for other genes, in a 20 ml reaction mixture. The RT-PCR products were electrophoresed on 1.5% agarose gel in TAE buffer, stained with ethidium bromide and photographed under UV light. The GAPDH gene was used as an internal control. The signal intensity of each RT-PCR product was estimated by Shimazu 9301-PC scanner (Tokyo, Japan).

Nitric oxide analysis

The nitric oxide was determined by measuring the amount of nitrite in the cell culture supernatant, using Griess reagent, according to the manufacturer's protocol. The BV-2 cells were stimulated with LPS (1 mg/ml) and test agents for 16 h, then briefly centrifuged, and 150 μl of the cell culture supernatant was mixed with 150 μl of Griess reagent, and incubated for 10 min at room temperature (light protected). The absorbance was measured using an ELISA reader at 540 nm, and compared to a standard calibration curve prepared from sodium nitrite (Ryu *et al.*, 2000).

Measurement of iNOS activity

The BV-2 cells were stimulated with LPS (1 μg/ml) for 16 h, and the cells washed twice with PBS. They were then incubated with test agents for 16 h. Cells were briefly centrifuged and 150 μl of the supernatant mixed with 150 μl of Griess reagent, and incubated 10 min at room temperature (light protected). The absorbance was measured using an ELISA reader at 540 nm, and compared to a standard calibration curve prepared from sodium nitrite (Ryu *et al.*, 2000).

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from treated microglia were prepared as follows. Cells (2×10^7) were treated with 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 1% NP-40) on ice for 4 min. After 10 min of centrifugation at 3,000 rpm, the pellet was resuspended in 50 μl of extraction buffer (20 mM HEPES, pH7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF) and incubated on ice for 30 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at -70 °C. Its protein concentration was determined with a protein assay reagent from Bio-Rad. Five microgram of the nuclear proteins were incubated with ^{32}P-labeled NF-κB or AP-1 probes on ice for 30 min and resolved on a 5% acrylamide gel as previously described (Kim *et al.*, 2004). For the supershift assay, antibodies against p65 or p50 subunits of NF-κB (Santa Cruz, CA) were co-incubated with the protein in the reaction mixture for 30 min at 4°C prior to adding the radiolabeled probe.

RESULTS

Effect of CHD on the production of NO in LPS-induced BV-2 cells

To understand anti-inflammatory effect of CHD against brain ischemia, its inhibitory effect in NO biosynthesis was investigated in LPS-stimulated BV-2 cells (Fig. 1). When BV-2 cells were treated with CHD before stimulation of LPS, CHD significantly inhibited the LPS-induced NO production, with IC_{50} value of 4.8 μg/ml. However, CHD at a concentration of 20 μg/ml did not inhibit iNOS enzyme activity of BV-2 microglia cells produced by the stimulation of LPS.

Effect of CHD on protein expression levels of iNOS and COX-2 in LPS-induced BV-2 cells

To understand the inhibitory mechanism of CHD in the NO biosynthesis in LPS-induced BV-2 cells, its effect on iNOS protein expression were examined (Fig. 2). Stimulation of the BV-2 cells with LPS-induced the iNOS protein expression, as

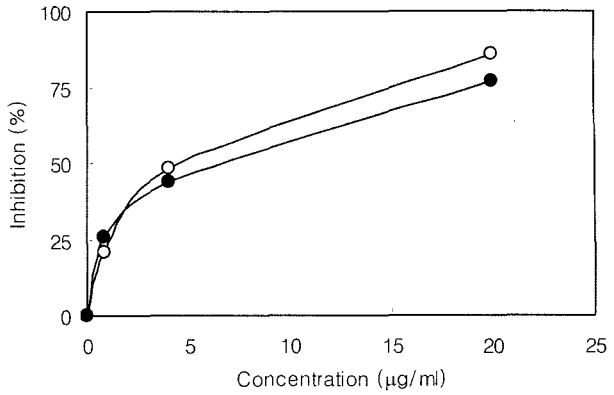

Fig. 1. Inhibitory effect of CHD (open circle) and prednisolone (closed circle) on NO production of LPS-stimulated BV-2 cells. LPS (1 μg/ml) for 16 h in the presence or absence of indicated concentrations of CHD or prednisolone was treated in BV-2 cells.

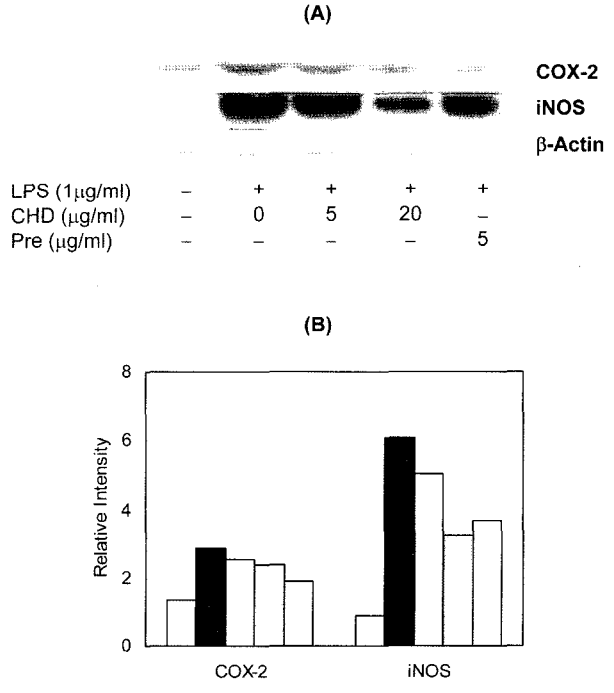

Fig. 2. Effect of CHD on protein expressions of iNOS and COX-2 in LPS-stimulated BV-2 cellls. (A) Immunoblot analysis. (B) Relative intensity of immunoblots (COX-1, COX-2, iNOS, IL-1β or TNF-α /GAPDH). □, normal control; ■, treated with LPS alone; ▨, treated with LPS and 5 μg/ml of CHD; ▦, treated with LPS and 20 μg/ml of CHD; ▧, treated with LPS and 5 μg/ml of prednisolone. LPS (1 μg/ml) for 16 h in the presence or absence of indicated concentrations of CHD or prednisolone (Pre) was treated in BV-2 cells.

determined by immunoblot analysis. CHD significantly reduced the levels of the iNOS protein expression. Densitometer scans of the respective blots showed that CHD at concentrations of 5 and 20 μg/ml reduced the levels of the iNOS protein by 22 and 55%, respectively, compared with the control cells stimulated with LPS. The inhibitory effect of CHD on iNOS protein expression correlates with the reduced amount of nitrite in BV2 microglial cells.

Stimulation of the BV-2 cells with LPS also induced the expression of the COX-2 protein. CHD at concentrations of 5 and 20 μg/ml reduced the levels of the COX-2 protein by 22 and 32%, respectively, compared with the control cells stimulated with LPS.

Effect of CHD on mRNA expression levels of iNOS and COX-2 in LPS-induced BV-2 cells

RT-PCR analysis was performed to investigate the effect of CHD on mRNA expression of iNOS, COX-1/2, and cytokines in LPS-stimulated BV-2 microglia (Fig. 3). We found that CHD significantly inhibited the LPS-induced iNOS mRNA expression. DO at concentrations of 5 and 20 μg/ml inhibited mRNA levels of iNOS by 16 and 39%, respectively. LPS also significantly induced COX-2 mRNA expression, whereas the expression of COX-1 was constitutive. CHD at concentrations of 5 and 20 μg/ml inhibited the mRNA expression of COX-2 by 17 and 22%, respectively.

When the effects of CHD on anti-inflammatory cytokines in LPS-stimulated BV2 cells, was examined, it also significantly inhibited the IL-1β and TNF-α mRNA levels.

Effect of CHD on transcription factors in LPS-induced BV-2 cells

To further dissect the anti-inflammatory mechanism of CHD in microglial cells, its effects on transcription factors NF-κB and AP-1, which are known to be involved in the regulation of the expression of a number of cytokines and iNOS in inflammatory reactions (Flora *et al.*, 2002; Kim *et al.*, 2004) were examined by EMSA (Fig. 4). CHD significantly repressed NF-κB DNA binding activity in LPS-induced BV2 cells. CHD significantly reduced activated NF-κB protein expression level in the nucleus as well as inhibited I-kB degradation in the cytoplasm (Fig. 4C). CHD also significantly repressed AP-1 DNA binding activities in LPS-induced BV2 cells (Fig. 5).

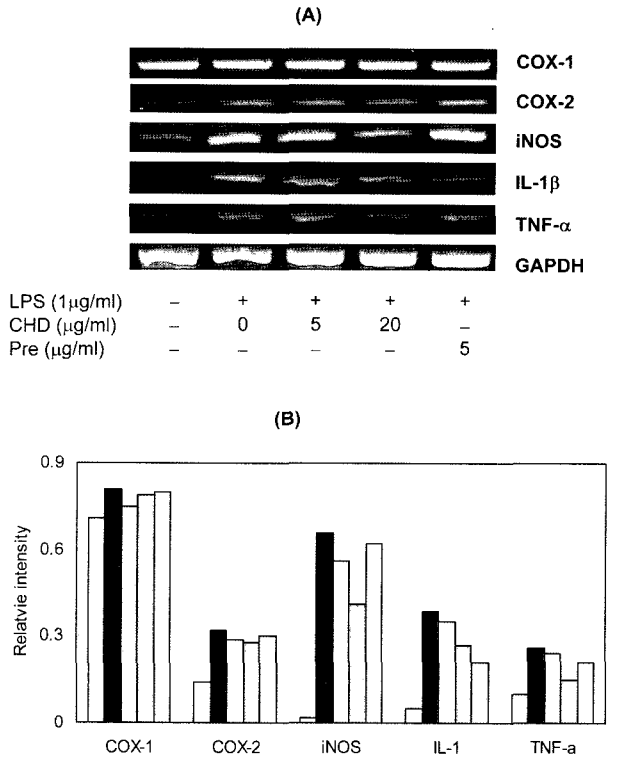

Fig. 3. Effect of CHD on mRNA expressions of iNOS, COX-2, IL-1β and TNF-α in LPS-stimulated BV-2 cellls. (A) RT-PCR products, (B) Relative intensity of RT-PCR products (COX-1, COX-2, iNOS, IL-1β or TNF-α /GAPDH). □, normal control; ■, treated with LPS alone; ▨, treated with LPS and 5 μg/ml of CHD; ▦, treated with LPS and 20 μg/ml of CHD; ▧, treated with LPS and 5 μg/ml of prednisolone. LPS (1 μg/ml) for 16 h in the presence or absence of indicated concentrations of CHD or prednisolone (Pre) was treated in BV-2 cells.

DISCUSSION

An inflammatory reaction in the brain, due to intervention or decrease of blood circulation, causes an ischemic stroke (Reynolds *et al.*, 2003; Lindsberg and Grau, 2003). The inflammatory reactions are modulated by macrophages as well as neutrophils. Constitutive and inducible NOS, COX-1 and COX-2 produce two pleiotropic mediators, NO and prostaglandins, at inflammatory sites and cause inflammation reactions (Dolan *et al.*, 2000). In the present study, CHD potently inhibited the NO biosynthesis of BV-2 cells induced by LPS. CHD inhibited the mRNA as well as protein expression levels of COX-2 and iNOS. However, CHD did not inhibit iNOS enzyme activity. CHD also inhibited the activation of NF-κB and AP-1 transcription factors, which are upstream modulators

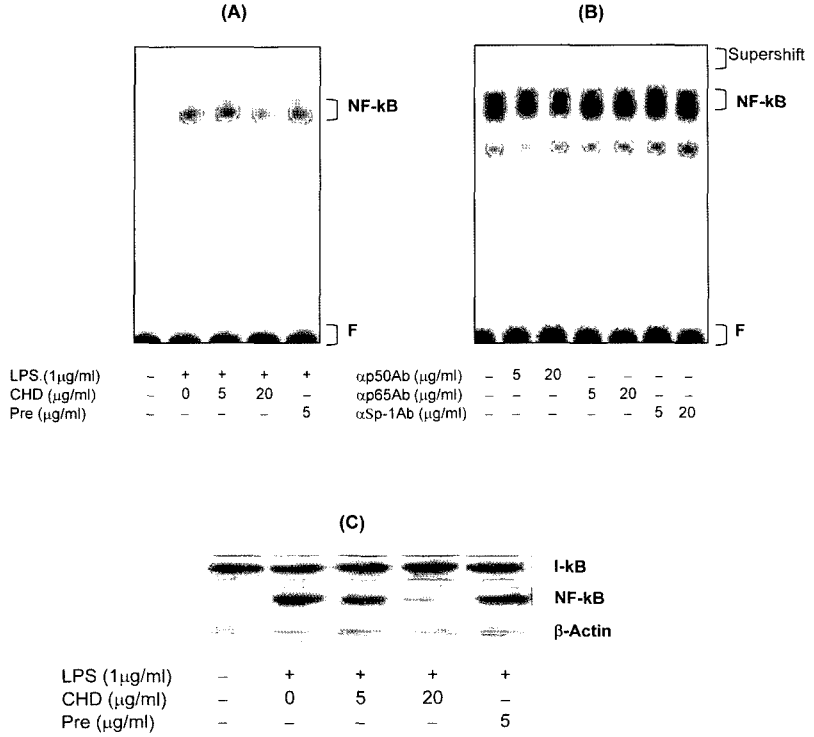

Fig. 4. Effect of CHD on NF-κB and AP-1 activities in activated microglia. (A) EMSA for NF-κB DNA binding activities. Nuclear extracts were prepared from BV2 cells after treatment with LPS (1 μg/ml) for 6 h in the presence or absence of indicated concentrations of CHD or prednisolone. NF-κB-DNA complex is indicated as bracket. (B) A supershift assay indicated that the NF-κB complex is composed of p65 and p50 subunits because DNA-protein complex of NF-κB was supershifted by antibodies to p65 and p50 subunit (right panel). Coincubation with Sp1 antibody did not generate supershift band. (C) Effect of CHD on activation of NF-κB in nuclei and I-κB in cytoplasm by immunoblot assay. LPS (1 μg/ml) for 16 h in the presence or absence of indicated concentrations of CHD or prednisolone (Pre) was treated in BV-2 cells.

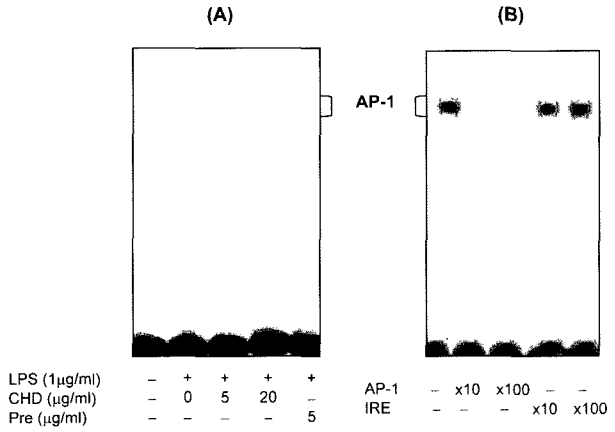

Fig. 5. Effect of CHD on AP-1 activities in activated microglia. (A) EMSA for AP-1 DNA binding activities. Nuclear extracts were prepared from BV2 cells after treatment with LPS (1 μg/ml) for 6 h in the presence or absence of indicated concentrations of CHD or prednisolone. The same nuclear extracts used in NF-κB EMSA were incubated with P^{32}-labeled AP-1 probe. (B) The DNA-protein complex indicated by the arrow was competed by cold AP-1, but not by IRE oligonucleotide, indicating that the complex is AP-1 sequence-specific.

of iNOS and COX-2 gene expression in LPS-stimulated BV2 microglial cells (Jongeneel, 1995; Lowenstein *et al.*, 1993). We previously reported that CHD could inhibit the protein expression of COX-2 and iNOS by the modulating NF-κB transcription factor in LPS-stimulated RAW264.7 cells (Cho *et al.*, 2004). These findings suggest that CHD may inhibit iNOS expression by the regulation of NF-κB and/or AP-1-related signal pathways. The anti-inflammatory effect of CHD may be through inhibition of both NF-κB and AP-1 activities.

The damage to brain neuronal cells due to an inflammatory reaction activates neutrophils and macrophages, which produce proinflammatory cytokines IL-1β and TNF-α (Lennard *et al.*, 2000; Flora *et al.*, 2002). In the present study, CHD significantly inhibited the expression levels of these cytokines, which is also transcribed by NF-κB and AP-1 transcription factors.

These findings suggest that CHD can downregulate NF-κB and AP-1 transcription factor activation pathways and may improve brain ischemia.

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